Glycobiology Insights



ORIGINAL RESEARCH

# Peptide Mimetics of Terminal Sugars of Complex Glycans

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**Abstract:** In this project we asked whether short peptides would mimic complex carbohydrate structures and express biological activity. Amino acid sequences were identified as potential glycan mimetics by molecular modeling of docking to binding sites of lectins. The sequence HPSLK was synthesized as a quadravalent structure from a tri-lysine scaffold. Solid-phase assays showed that this peptide bound strongly to lectins from *Triticus vulgaris* (wheat germ agglutinin, WGA) and *Dolichos biflorus* (DB), which bind monosaccharides, and to lectins from *Sambucus nigra* (SNA1) and *Maackia amurensis* (MAA), which are specific for oligosaccharides with terminal 5-acetylneuraminic acid-galactose sequences. Further modeling led to synthesis of a longer peptide, NPSHPSLG, along with a variant, NPSHPLSG, as quadravalent structures. The longer peptides bound weakly, if at all, to WGA and DB but bound strongly to SNA1 and MAA. Mucin inhibited binding of HPSLK to WGA and DB, while fetuin inhibited binding of NPSHPSLG and NPSHPLSG to SNA1 and MAA. These results suggest that the peptides interact with the lectins at glycan-binding sites. When tested for biological activity, the peptides stimulated internalization of opsonized microspheres, which was blocked by wortmannin, an inhibitor of phagocytosis. Whereas HPSLK stimulated phosphorylation of the signaling proteins STAT2 and STAT6, NPSHPSLG and NPSHPLSG stimulated phosphorylation of STAT6, a marker for the alternative activation of phagocytosis, more strongly than STAT2. Our results suggest that these synthetic peptides may be useful as biological response modifiers.

**Keywords:** lectins, glycomimetics, molecular modeling, peptide mimetics, phagocytosis

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## Introduction

Cells of the immune system express an extensive array of cell surface receptors that interact with glycans as regulatory ligands.[1] Peptide mimetics of the natural ligands have potential advantages as cellular response modifiers because of the large number of short sequences of amino acids that are possible, the well developed technology for large scale chemical synthesis, ease of purification, and stability. Peptide-based structures can be constructed that also bind with much higher affinities to lectins than glycan ligands.[2] A number of peptides that mimic sugars have been identified, some of which closely resemble specific sugars[3–5] and others that act as more general mimetics.[6] We previously identified several short peptide sequences by molecular modeling, which were incorporated into multivalent structures that bound to several lectins with high affinity.[7,8] Multivalency of ligands is required for high affinity interactions[9–11] and to facilitate cross-linking of receptors, which is often required for activation of cellular responses.[12]

Well-characterized lectins were used as receptor analogs in our study to analyze the mimetic properties of peptides. In binding assays, a quadravalent peptide with the sequence His-Pro-Ser-Leu-Lys (HPSLK) had characteristics of a general sugar mimetic and bound to several lectins with higher affinity than monosaccharide ligands such as 5-acetylneuraminic acid (Neu5Ac) or N-acetylgalactosamine (GalNAc). In contrast, longer peptide analogs, in particular Asn-Pro-Ser-His-Pro-Ser-Leu-Gly (NPSHPSLG) and a variant Asn-Pro-Ser-His-Pro-Leu-Ser-Gly (NPSHPLSG), did not bind significantly to these lectins but bound strongly to lectins specific for complex glycans that terminate with Neu5Ac-galactose (Gal). Nanomolar concentrations of the peptides stimulated uptake of opsonized microspheres by adherent cells in cultures of human peripheral blood mononuclear cells (PBMCs).[8] The data reported here extend our previous studies on the mimetic character of these peptides.

## Methods and Materials

### Peptide design and synthesis

Unique peptide sequences were designed by molecular modeling of docking to sugar-binding sites of lectins, downloaded from the Protein Data Bank (PDB), with ArgusLab 4.0.1 software (Mark A. Thompson, Planaria Software LLC, Seattle, WA, http://www.arguslab.com). Quadravalent peptides were synthesized on a tri-lysine core[13,14] utilizing Fmoc (9-fluorenylmethoxycarbonyl)-protected amino acids and a Milligen Biosearch 9050+ continuous flow peptide synthesizer (Millipore, Billerica, MA). The C-terminus consisted of either an amide group (no tag) or ε-biotinyl-lysine. The structure for the HPSLK peptide, with a C-terminal amide, was $[(HPSLKGGGS)_2K]_2K\text{-}NH_2$. The sequence GGGS was included as a spacer to extend the active sequence from the tri-lysine core. Peptides were also synthesized with similar structure for the sequences NPSHPSLG and NPSHPLSG. After peptides were cleaved from the resin and dried, 200 to 300 mg were dissolved in water, neutralized with $Na_2CO_3$, applied to a column (1 × 5 cm) of CM-Sephadex C-50, and washed extensively with water to remove side-products of synthesis. Peptides were eluted with 0.1 N HCl, and then purified on a preparative Jupiter Proteo C12 column (21.2 × 250 mm) (Phenomenex, Torrance, CA) using a gradient of acetonitrile in water containing 0.1% trifluoroacetic acid. Eluted peptides were dried, dissolved in water, neutralized to pH 5, and passed through a DEAE-Sephadex A-25 column (1 × 30 cm) to remove trifluoroacetate and endotoxin, diluted with endotoxin-free 100 mM NaCl, and filter-sterilized. Concentration was determined by the bicinchoninic acid assay (Pierce, Rockland, IL). Correct synthesis and purity were analyzed by mass spectroscopy and amino acid sequence analysis.

### Lectin binding

For lectins available as peroxidase conjugates, biotin-tagged peptides were added to streptavidin-coated wells of a microtiter plate (binding capacity, 125 pmoles per well, Pierce) and incubated 1 h at room temperature. The wells were washed, blocked with 1% gelatin in 50 mM Tris-HCl (pH 7.5) containing 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM $MnCl_2$ (buffer A) and washed two times with buffer A. Then 50 µl of 1 µg/ml horseradish peroxidase-conjugated lectins (Sigma-Aldrich, St. Louis, MO) in buffer A were added. After 1 h incubation, wells were washed 4 times with buffer A and then 50 µl of peroxidase substrate (1-Step Ultra TMB-ELISA, Pierce) were added. Two to 10 min later the reaction was stopped with 50 µl 2 M $H_2SO_4$ and absorbance was read immediately at 450 nm. The amount of lectin bound was calculated from the specific activity of the peroxidase-conjugates ($OD_{450}$/min/ng protein).

The protocol was modified to assay binding of peptides to unconjugated lectins from *Sambucus nigra* (SNA1) and *Maackia amurensis* (MAA). Lectin-coated microwell strips (AlerCHEK, Portland, ME) were hydrated in buffer A, blocked with 1% gelatin in buffer A, and then biotinylated peptide was added to each well. After 1 h incubation, the wells were washed 3 times with buffer A and then 50 µl of 0.3 µg/ml peroxidase-conjugated streptavidin (Sigma-Aldrich) were added. Wells were washed 4 times with buffer A and peroxidase activity was assayed as above.

Fetuin (Calbiochem, La Jolla, CA) was digested with recombinant α-(2→3,6,8,9)-neuraminidase from *Arthrobacter ureafaciens* and β-(1→3,4,6)-galactosidase, a mixture from *Streptococcus pneumoniae* and *Xanthomonas sp.*, which were obtained from Sigma-Aldrich and used according to the supplier's instructions.

## Phagocytosis assay

Phagocytic activity was assayed as described previously.[8] Briefly, streptavidin-coated microspheres, dyed with Dragon Green (0.97 µm diameter, Bangs Laboratories, Inc., Fishers, IN), were opsonized with rabbit anti-streptavidin serum (Sigma-Aldrich) and washed with phosphate buffered saline, pH 7.4 (PBS). Human PBMCs were cultured in microtiter plates in RPMI-1640 medium containing 10% fetal bovine serum (FBS) and 1:100 dilution of penicillin-streptomycin solution (Mediatech, Inc., Herndon, VA). Cultures were washed to remove non-adherent cells and fresh medium containing FBS, antibiotics, and 50 nM peptide was added to adherent cells for an additional for 20 to 24 h. Microspheres were added at approximately a 10:1 ratio to total cells, and after an additional 30 to 60 min of incubation, formalin was added to a concentration of 2%. The samples were allowed to stand at 4 °C overnight, then washed 3 times with PBS to remove free microspheres and examined with an inverted microscope and a 40X objective lens. Fluorescent images were captured by Metamorph software (Molecular Dynamics, Sunnyvale, CA) with a Nikon inverted microscope and a 40X objective lens.

## Signal transduction

PBMCs in RPMI-1640 medium, supplemented with 2 mM glutamine and 0.1% ovalbumin (Sigma-Aldrich), were plated at a density of $1 \times 10^5$ cells (250 µl) on 0.45 µm MultiScreen HTS HV sterile filter plates (Millipore) and incubated overnight at 37 °C, 5% $CO_2$. Peptides were added to 50 nM final concentration and incubated for 10 min. Phosphorylation of STAT2 and STAT6 was measured with a FACE STAT kit (Active Motif, Carlsberg, CA) according to the supplier's instructions. Briefly, the incubation was stopped by fixation with 25 µl of 37% formaldehyde (4% final concentration) for 20 min. Fixed cells were washed 3 times with PBS containing 0.1% Triton X-100 (wash buffer) and then incubated with 100 µl wash buffer containing 1% $H_2O_2$ and 0.1% azide for 20 min to inactivate cellular peroxidase activity. Non-specific binding sites were blocked with 100 µl of 3% bovine serum albumin in PBS for 1 h. Primary antibody, at a 1:500 dilution, was incubated with cells overnight at 4 °C and the wells were then washed 2 times. Peroxidase-conjugated secondary antibody, at a 1:2000 dilution, was added and incubated for 1 h at room temperature. Wells were washed 4 times and then 100 µl peroxidase substrate (Active Motif) was added. The reaction was stopped with 100 µl 2 M $H_2SO_4$ when sufficient blue color had developed. Absorbance was read immediately at 450 nm with a microtiter plate reader. Primary antibodies are specific for phosphorylated tyrosine-689 of STAT2 or phosphorylated tyrosine-641 of STAT6. Antibodies for total STAT2 and STAT6 recognize the proteins regardless of phosphorylation state.

# Results

## Identification of a sugar mimetic

Short peptides, 5 to 8 amino acids in length, were screened as potential mimetics by molecular modeling of docking to crystal structures of sugar-specific lectins. Amino acid residues that comprise the binding site of a lectin were selected from the literature that describes each lectin. The *in silico* experiments suggested that a peptide with the sequence HPSLK would bind to a variety of lectins and with a range of affinities. These lectins (PDB accession number and predicted binding energy) were the GalNAc/Gal-specific lectin from *Helix pomatia* (2CE6, –4.24 kcal/mol),[15] the Gal-specific lectin from *Griffonia simplicifolia* I-$B_4$ (1GNZ, –6.56 kcal/mol),[16] the lectin from *Triticus vulgaris* (1WGT and 7WGA, –8.22 kcal/mol),[17–19] which binds

GlcNAc and Neu5Ac but also clusters of GalNAc,[20,21] the GalNAc-specific lectin from *Vicia villosa* (1N47, –9.22 kcal/mol),[22] and the GalNAc-specific lectin from *Dolichos biflorus* (1LU2, –10.0 kcal/mol).[23] Correspondence between predicted binding energy and extent of binding to these lectins was observed for this peptide.[8] We chose the lectins from *Triticus vulgaris* (wheat germ agglutinin, WGA) and from *Dolichos biflorus* (DB) for further study because modeling predicted that both lectins should bind this peptide with a relatively high binding energy.

## Peptide binding to lectins

The monovalent biotinylated peptide HPSLK was synthesized by extension from a spacer sequence, GGGS, attached to the α-amino group of an ε-biotinyl-lysine amide residue. The concept of affinity as a function of ligand density[9–11] led to synthesis also as bivalent or quadravalent structures. The bivalent molecule contained the peptide sequence extended from the α and ε amino groups of a lysine residue linked to ε-biotinyl-lysine amide. The quadravalent molecule contained the peptide sequence extended from the four amino groups of a tri-lysine scaffold[14] that was linked to ε-biotinyl-lysine amide. The effect of valency was tested in a solid-phase assay in which the C-terminal ε-biotinyl-lysine anchored the peptide to streptavidin that was bound in microtiter plate wells. This arrangement should allow maximal flexibility of the peptides for interaction with lectins. The extent of subsequent binding of lectins was detected by peroxidase conjugated to the lectins. To achieve equal numbers of HPSLK sequences, 25 pmoles of the quadravalent peptide, 50 pmoles of the bivalent peptide, and 100 pmoles of the monovalent peptide were added per well. As presented in Figure 1, with seven different lectins the quadravalent peptide bound approximately two times more lectin than the bivalent peptide, which bound five times more than the monovalent peptide. Peptides without the biotin tag were not retained in the assay.

A longer peptide, NPSHPSLG, was also synthesized as a quadravalent analog of HPSLK. The "core" sequence HPSL was retained, P was included in the second position to reduce aminopeptidase activity,[24,25] and the potential trypsin cleavage site at the C-terminus of the active sequence was removed by replacing K with G. As shown in Figure 2, in contrast

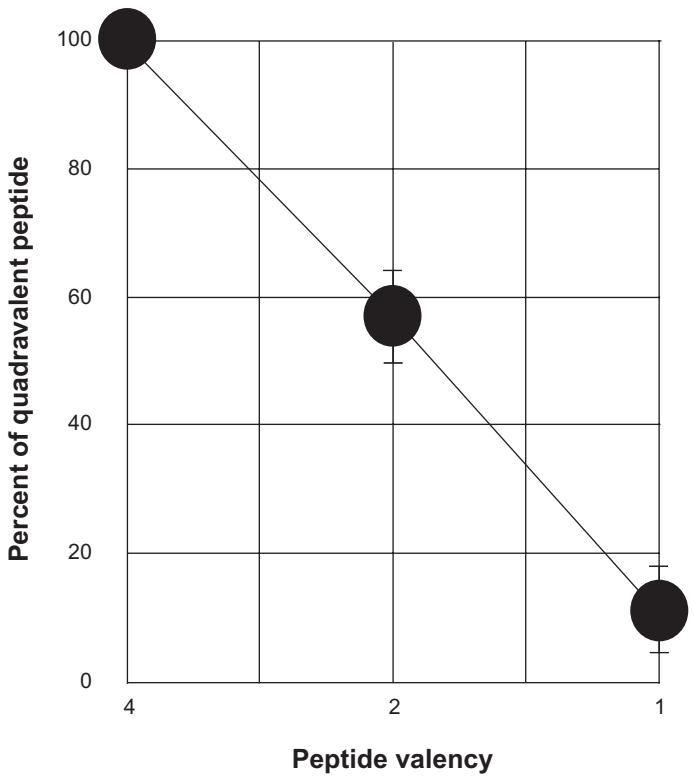


**Figure 1.** Binding of bivalent or monovalent HPSLK peptides to lectins as compared with that of the quadravalent peptide, which was set as 100%. The assay contained 25 pmoles of the quadravalent peptide, 50 pmoles of the bivalent peptide, and 100 pmoles of the monovalent peptide to provide an equal number of HPSLK sequences per well. The data points are the mean ± SD from measurements with seven different lectins. The lectins (and the extent of binding of each to the quadravalent peptide) were *H. pomatia* (0.15 ng), *G. simplicifolia* I-$B_4$ (0.57 ng), *D. biflorus* (2.0 ng), WGA (1.6 ng), SNA1 (1.7 ng), MAA (1.9 ng) and concanavalin A (3.2 ng).

to HPSLK, the quadravalent longer NPSHPSLG did not bind significantly to WGA or DB. A similar peptide, NPSHPLSG, in which the SL sequence was reversed, had similar properties.

We then assayed binding to two additional lectins, SNA1 from *Sambucus nigra*[26] and MAA from *Maackia amurensis*,[27,28] which bind preferentially to di- and trisaccharides rather than monosaccharides. For these assays, the lectins were fixed in wells of microtiter strips. After adding biotinylated peptide, and washes to remove unbound peptide, streptavidin conjugated with peroxidase was used to detect the amount of bound peptide. NPSHPSLG and NPSHPLSG, in addition to HPSLK, bound strongly to SNA1 and MAA (Fig. 2). Strong binding to these lectins was confirmed by the assay in which peroxidase-conjugated lectins were incubated with peptide bound to streptavidin (see Fig. 6). SNA1 requires a terminal disaccharide

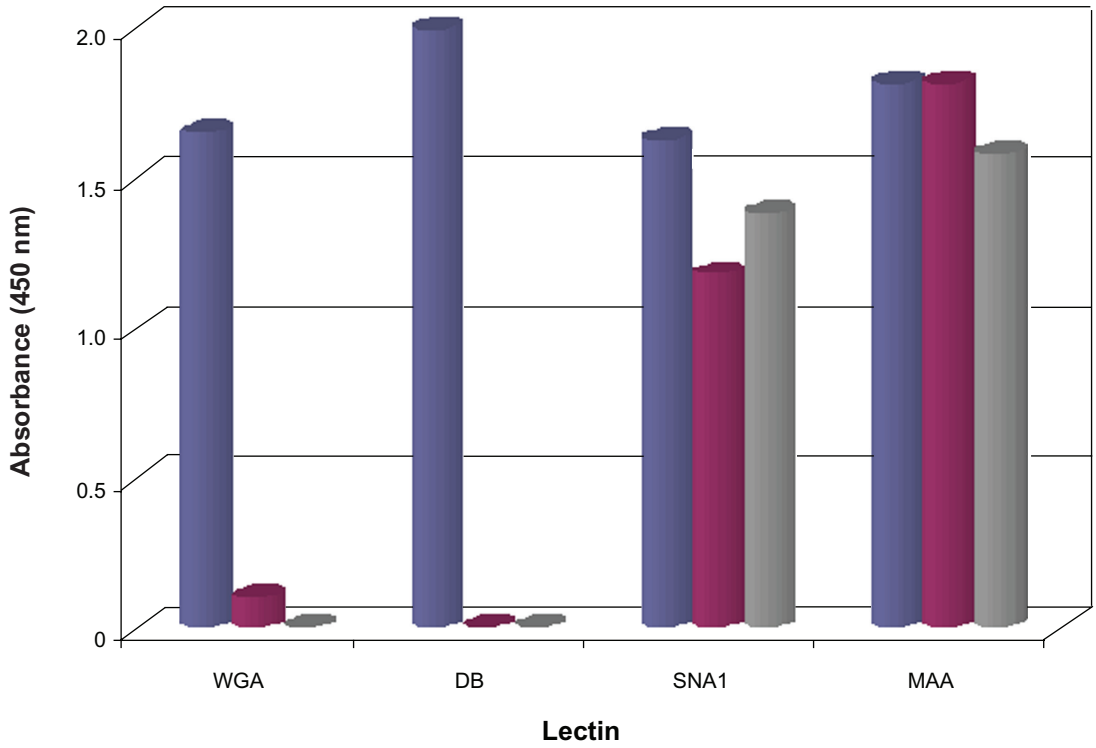


**Figure 2.** Binding of quadravalent peptides to lectins. Binding of WGA and DB was assayed by peroxidase-conjugated lectins added to peptide bound to streptavidin. For SNA1 and MAA, peptides were added to bound lectins and the extent of binding was detected with peroxidase-conjugated streptavidin. Blue, HPSLK; magenta, NPSHPSLG; grey, NPSHPLSG. The experiment was repeated in quadruplicate three times with similar results.

with the structure Neu5Ac(α2-6)Gal/GalNAc-[26] while MAA requires three intact terminal sugars with the sequence Neu5Ac(α2-3)Gal(β1-4)GlcNAc/Glc-.[27]

From assays of additional peptides, we found that binding of the quadravalent structure with the sequence Val-Ser-Asn-Gln-His (VSNQH) to SNA1 and MAA could not be detected above blank values (data not shown). This sequence was therefore chosen as a control (inactive) peptide.

Binding of the core peptide HPSLK and the core variant NPSHPLSG to SNA1 and MAA was examined as a function of the amount of peptide added to assay wells. As shown in Figure 3, binding of HPSLK and NPSHPLSG to SNA1 was saturated at about 50 pmoles peptide in the assay, with half-maximal binding at 5 to 10 pmoles per well. Higher amounts of peptide, about 100 pmoles, were required to saturate the assay with MAA, with half-maximal binding obtained near 25 pmoles peptide per well (Fig. 4).

## Competition with glycoproteins

To confirm that the peptides interacted with carbohydrate-binding sites on the lectins, competition binding assays were performed. As described previously,[8] binding of HPSLK to WGA was inhibited by high concentrations of Neu5Ac, which provided an approximate $K_d$ of 7 nM for the peptide. Insufficient inhibition of binding of the

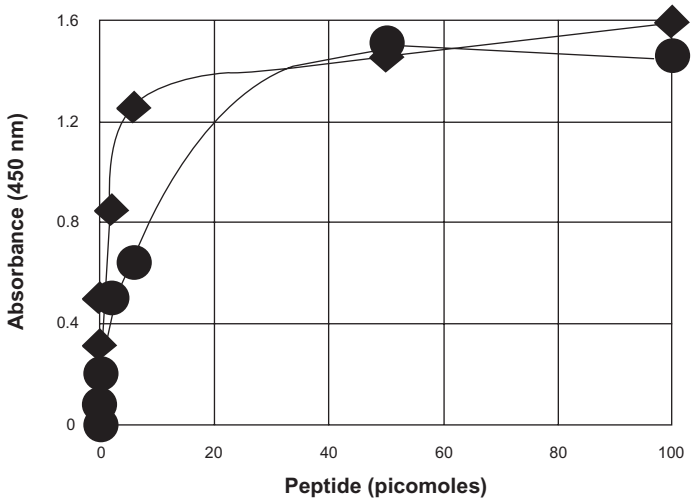


**Figure 3.** Binding of (◆) HPSLK and (●) NPSHPLSG to SNA1 as a function of the amount of peptide added to wells containing bound lectin. Symbols indicate average values of three experiments done in quadruplicate.

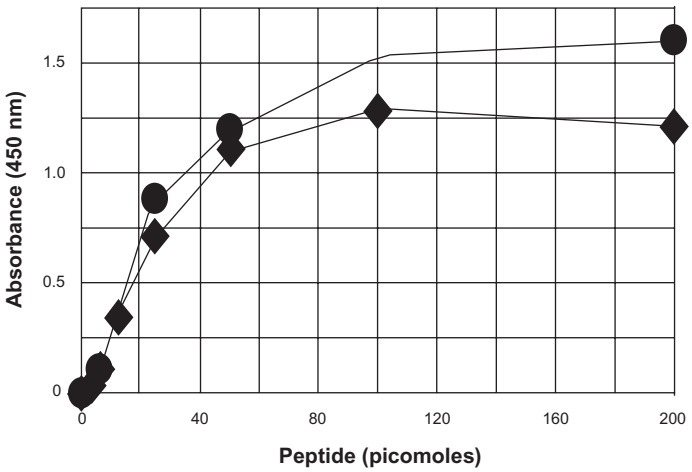


**Figure 4.** Binding of (◆) HPSLK and (●) NPSHPLSG to MAA as a function of the amount of peptide added to wells containing bound lectin. Symbols indicate average values of three experiments done in quadruplicate.

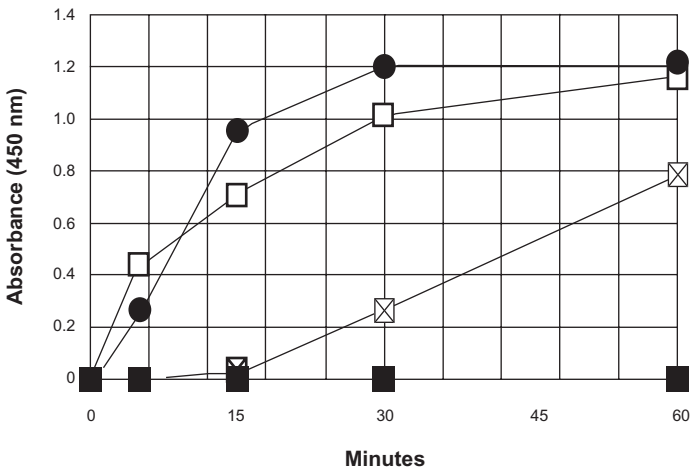


**Figure 5.** Time course of binding of WGA to HPSLK and inhibition of binding by mucin. Mucin was added at several concentrations along with the lectin to 100 pmoles peptide bound to streptavidin. (●) No mucin; (□) 12.5 μg/ml mucin; (⊠) 25 μg/ml mucin; (■) 50 μg/ml mucin. The assay was done tthree times with similar results.

peptide by GalNAc was obtained with DB to calculate a $K_d$. We then tested the ability of porcine stomach mucin to compete with HPSLK binding to WGA and DB. Mucin is a multivalent glycoprotein with a variety of glycans with terminal GalNAc, Gal, GlcNAc, fucose and a minor amount of Neu5Ac.[29,30] Included among these terminal sugars are those to which WGA and DB are relatively specific. Binding of HPSLK to DB was essentially completely inhibited by 50 μg/ml mucin, described by a typical concentration-dependent curve with 50% inhibition at 20 μg/ml mucin (data not shown). Only partial inhibition was obtained with WGA after an hour of incubation. To further explore the inhibition of binding to WGA, the time course of the assay was examined. While insignificant inhibition was obtained with 12.5 μg/ml mucin, added to 100 pmoles peptide, strong inhibition was found with 25 μg/ml mucin during the first 15 min of incubation, before maximal binding of the lectin was achieved (Fig. 5). Thereafter, the peptide appeared to displace mucin from the lectin. Complete inhibition was obtained within the 30 min time period with 50 μg/ml mucin. Only slight inhibition was found for binding of the peptides to SNA1 and MAA, possibly because of the paucity of Neu5Ac (*ca*. 1%) on this glycoprotein (data not shown).

Fetuin contains collectively 12 to 15 oligosaccharides that terminate predominantly as Neu5Ac-Gal, with nearly equal α2-3 and α2-6 linkages, on three N-linked and three O-linked glycans per molecule.[31–34] If indeed NPSHPSLG and NPSHPLSG mimic Neu5Ac-Gal termini, fetuin should compete effectively with the peptides and inhibit their binding to these lectins. As shown in Figure 6, essentially complete inhibition of binding of NPSHPSLG and NPSHPLSG to SNA1 and MAA was achieved with 500 pmoles of fetuin per well (250 μg/ml), with about 50% inhibition at

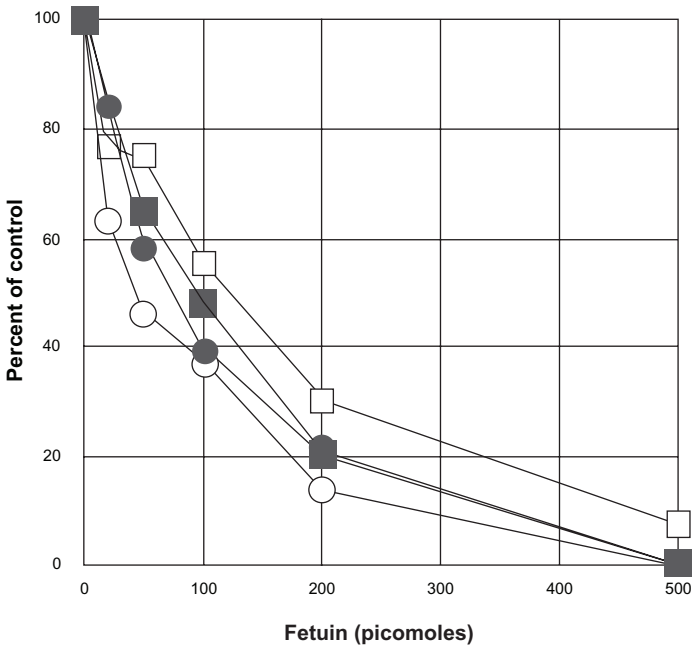


**Figure 6.** Inhibition of binding of NPSHPSLG and NPSHPLSG to SNA1 and MAA by fetuin. (●), (■) Binding of NPSHPSLG to SNA1 and MAA, respectively. (○), (□ ) Binding of NPSHPLSG to SNA1 and MAA, respectively. Peroxidase-conjugated lectins were added to wells along with the indicated amount of fetuin to 100 pmoles of peptides bound to streptavidin. The experiment was repeated three times with similar results. Lectin bound in the absence of fetuin was assigned a value of 100%.

100 pmoles. These results indicate that the peptides bind the lectins at least as strongly as a natural multivalent glycan structure.

To further examine the ability of fetuin to inhibit binding of peptides to SNA1 and MAA, concentrations were chosen that were one-third, equal or 5-fold the molar concentration of peptide in the assay. Only slight inhibition by fetuin was found in assays of binding of HPSLK to SNA1 (Fig. 7A) or MAA (Fig. 7B). Fetuin strongly inhibited binding of the longer peptides to SNA1 and MAA. To confirm that fetuin inhibited binding by displacing the peptide from a glycan-binding site, the glycoprotein was digested with α-neuraminidase. Fetuin thus depleted of Neu5Ac was added at 10-fold the molar concentration of peptide. Binding of NPSHPLSG to MAA was completely restored (Fig. 7B) and nearly completely to SNA1 (Fig. 7A). Partial inhibition remained with NPSHPSLG with both lectins. Additional digestion with β-galactosidase eliminated inhibition of binding of HPSLK and lessened inhibition of binding of NPSHPSLG to MAA. Enzymatic digestion may have been incomplete, although the lack of inhibition of binding of NPSHPLSG by fetuin after incubation with the enzymes suggested that substantial removal of Neu5Ac had occurred. These experiments suggested that NPSHPSLG and NPSHPLSG were interacting with the lectins at glycan-binding

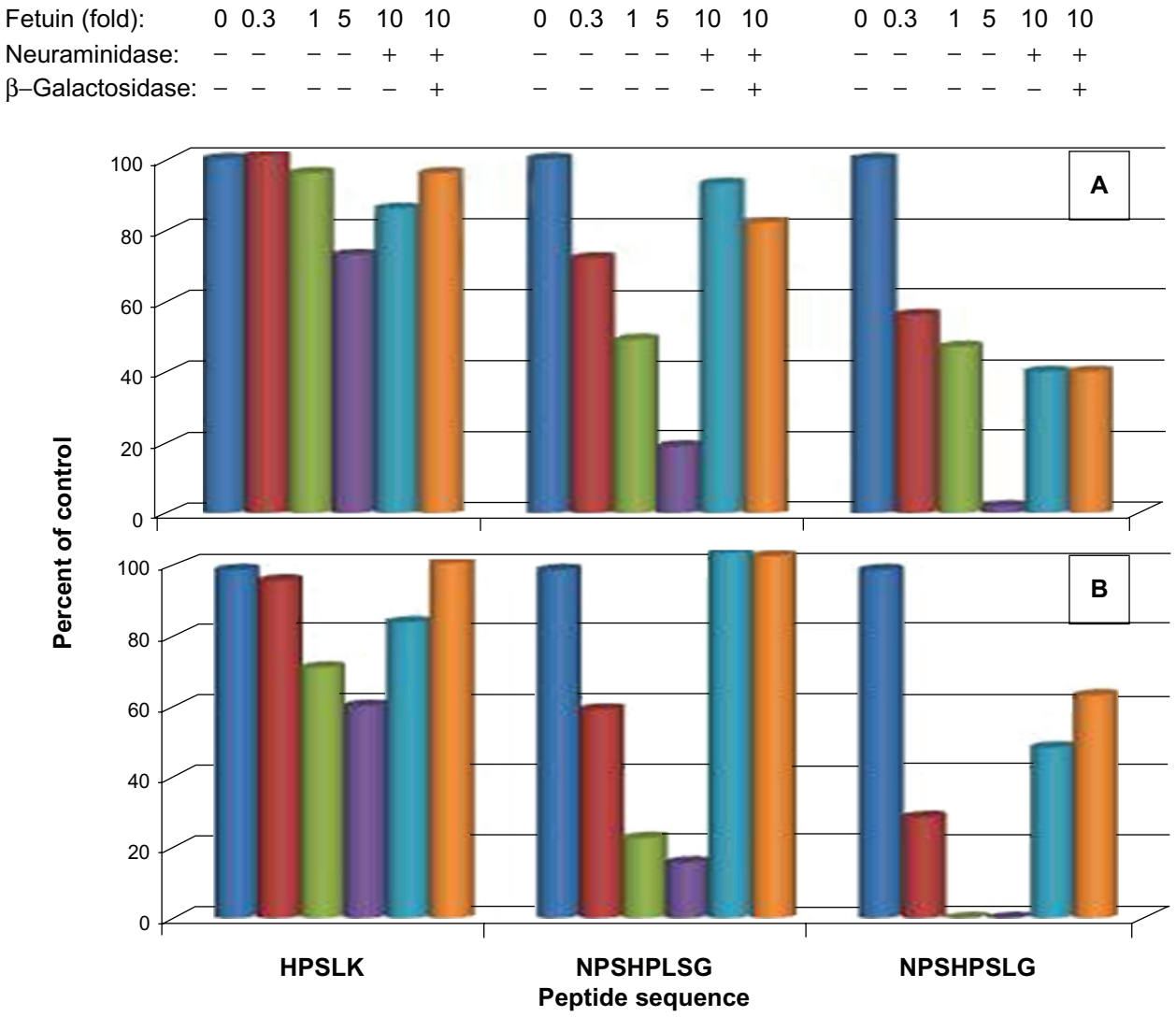


**Figure 7.** Inhibition of binding of peptides to (A) SNA1 and (B) MAA by fetuin and after digestion of fetuin with α-neuraminidase and β-galactosidase. The table at the top of the figure indicates the amount of fetuin added relative to the peptides, of which 100 pmoles were added to each well. The assay was done in quadruplicate.

sites, with NPSHPLSG the stronger Neu5Ac-Gal mimetic.

## Stimulation of cellular responses

Cells of the immune system contain glycan-binding proteins on their surface that regulate innate and adaptive immune functions.[1] A family of receptors on these cells, designated Siglecs (sialic-acid-binding immunoglobulin-like lectins), interact with glycans that have terminal Neu5Ac-Gal and trigger internalization of pathogens.[35] We explored the ability of the three peptides used in this study to stimulate phagocytosis. In addition, to determine whether uptake of opsonized microspheres occurred by endocytosis or Fcγ receptor-mediated phagocytosis, samples were treated with 100 nM wortmannin, an inhibitor of phagocytosis but not of endocytosis.[36] Control samples received either vehicle or the quadravalent peptide with the sequence VSNQH, which did not detectably bind to lectins. Cells in these cultures internalized few microspheres, and the number of which was not significantly different in cells treated with wortmannin (Figs. 8A, B). In contrast, microspheres were abundant in cells treated with HPSLK, NPSHPLSG and NPSHPSLG (Figs. 8C, D, E, columns 1 and 2). The peptide-treated samples incubated with wortmannin contained few microspheres and were similar in appearance to control samples (Figs. 8C, D, E, column 3).

To establish that the bead-like material within cells was indeed internalized microspheres, cells were examined by fluorescence microscopy. As shown in Figure 9A, cells treated with HPSLK contained dark material in phase contrast images (left panel) that was highly fluorescent (right panel). In cultures treated with wortmannin, fluorescent microspheres were detected only in the medium (Fig. 9B, arrows). Similar images were obtained with cells treated with NPSHPLSG (Figs. 9C, D) and NPSHPSLG (Figs. 9E, F). Cells treated with the control peptide, VSNQH, did not contain fluorescent material (Fig. 9G).

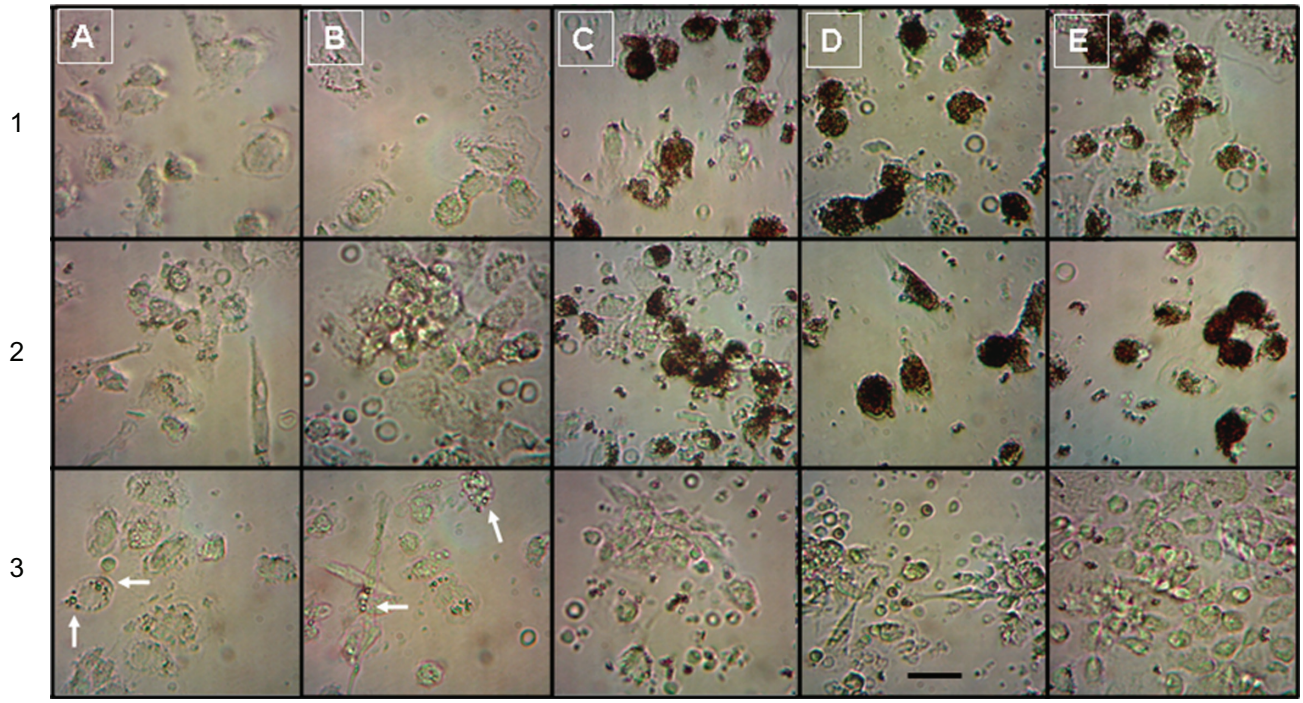


**Figure 8.** Phagocytosis of opsonized microspheres by peptide-treated cells. Non-adherent cells in PBMCs were removed after 24 h in culture and fresh medium was provided to adherent cells. Cells were incubated with 50 nM peptides for 20 h, with microspheres for 60 min, and then fixed with 2% formalin as described under Methods. Free microspheres were removed by washing 3 times with PBS and the samples were examined by microscopy. (**A**) Control samples without peptide. Cultures were treated with peptides (**B**) VSNQH; (**C**) HPSLK; (**D**) NPSHPLSG or (**E**) NPSHPSLG. Panels 1 and 2 in each row show two images from each sample; panel 3 shows samples treated with 100 nM wortmannin. Cells in (A) and (B) appear to contain a few beads near the surface, which were also visible in wortmannin-treated samples (arrows). Wortmannin dramatically reduced the number of beads in peptide-treated cells. Bar = 20 μm for all panels. This experiment was done three times with similar results.

A major pathway that regulates the immune system functions, including phagocytosis, through stimulation of phosphorylation of the signaling proteins STAT1 to STAT6.[37] JAK/STAT diverges into an *inflammatory* pathway that involves Janus kinase 1 (JAK1), which phosphorylates STAT1 and STAT2, and an alternative, *non-inflammatory* pathway of macrophage activation that involves Janus kinase 3 (JAK3), which phosphorylates STAT6.[38] Increased phosphorylation of these signaling proteins occurs within minutes after cells are treated with regulatory factors.[37] To determine whether these pathways are stimulated in cells treated with the peptides, we assayed phosphorylated epitopes in STAT2 and STAT6 with specific antibodies after a 10-min incubation with PBMCs. Our results showed that, consistent with the more general binding of HPSLK to lectins (Fig. 2), this peptide induced an increase in phosphorylation of STAT2 (Fig. 10A) and STAT6 (Fig. 10B). In contrast, NPSHPSLG and NPSHPLSG stimulated phosphorylation of STAT6 but not significantly of STAT2.

## Discussion

In this project we asked whether short peptides would mimic complex carbohydrate structures and express biological activity. As shown in Figure 2, quadravalent HPSLK bound strongly to WGA, DB, SNA1 and MAA, illustrating its ability to act as a

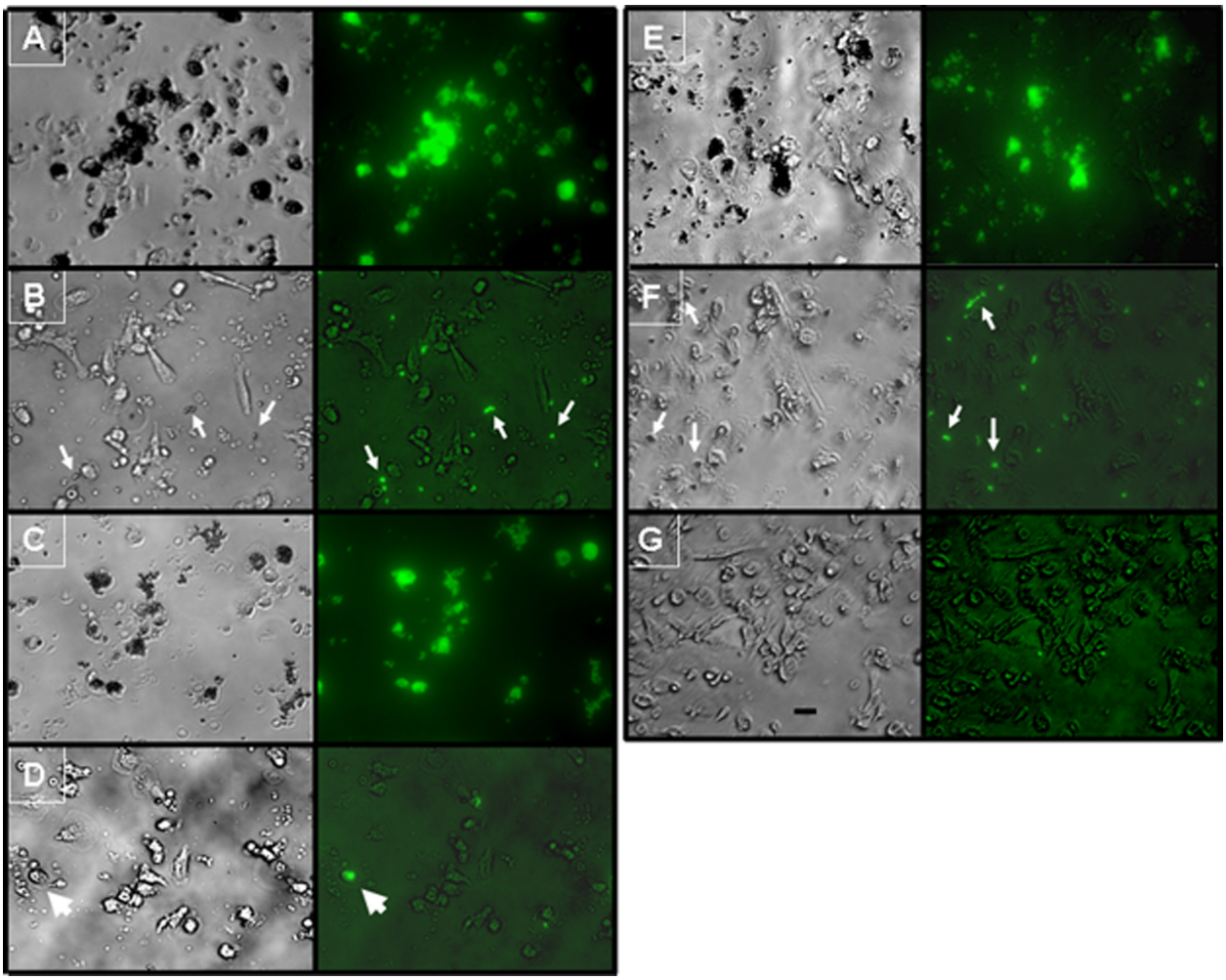


**Figure 9.** Fluorescence microscopy of cells treated with peptides. Cultures were incubated with 50 nM peptide for 24 h and then challenged with microspheres for 30 min. Cells were fixed with 2% formalin as described in the legend to Figure 8 and washed 3 times with PBS. Cells were examined by phase contrast microscopy (left panels) and fluorescence microscopy with blue excitation light (right panels). (**A**) HPSLK; (**B**) HPSLK plus wortmannin; (**C**) NPSHPLSG; (**D**) NPSHPLSG plus wortmannin; (**E**) NPSHPSLG; (**F**) NPSHPSLG plus wortmannin; (**G**) VSNQH. A few microspheres remained in the extracellular medium in wortmannin-treated cultures (arrows), and cells were occasionally found in these cultures that had internalized microspheres (D, arrowheads). Bar (in panel G) = 20 μm for all panels.

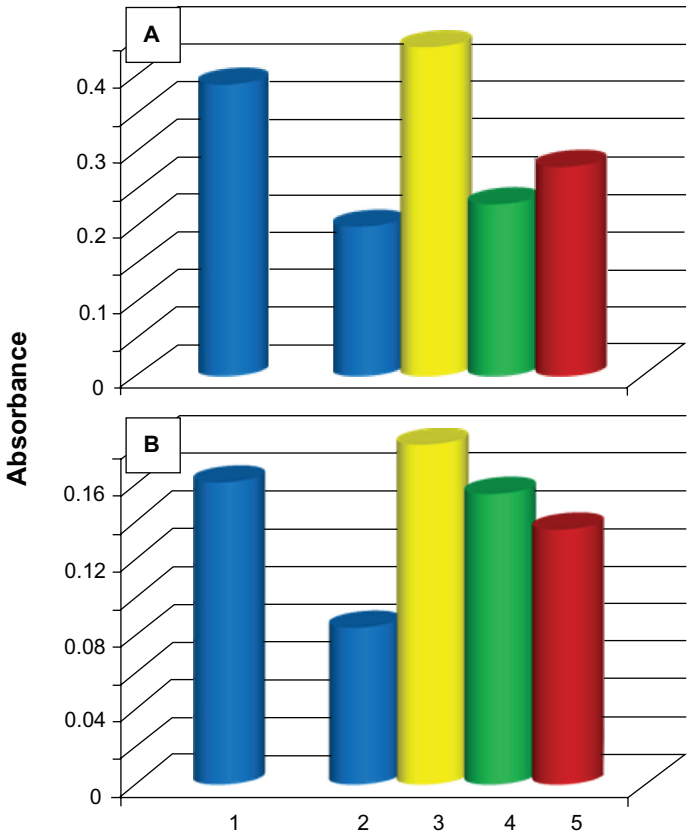


**Figure 10.** Phosphorylation of STAT2 and STAT6 in response to incubation with peptide. PBMCs were cultured in filter plates without serum overnight. Cells were then treated with 50 nM peptide for 10 min and fixed. Panel A, STAT2; panel B, STAT6. In each panel, the blue bars represent: (1) untreated total STAT2 or STAT6 or (2) phospo-STAT2 or -STAT6 in untreated cultures. Phosphorylated STAT2 (panel A) or STAT6 (panel B) is represented by bars for cells treated with HPSLK (3, yellow); NPSHPLSG (4, green) or NPSHPSLG (5, red). Each was assayed in duplicate using antibodies against total or phosphorylated STAT proteins.

general glycan mimetic. Interestingly, an analog of this sequence, NPSHPSLG, which retained the putative core sequence HPSL, and a variant of the longer sequence, NPSHPLSG, bound weakly, if at all, to lectins such as WGA and DB that bind monosaccharides. In contrast, these peptides bound strongly to SNA1 and MAA, which are specific for complex glycans. Thus, extending the sequence by three amino acids and modifying the core appeared to dramatically enhance specificity of binding to lectins. These experiments demonstrate that the sequence of the peptides, as well as valency, is critical for high affinity to the lectins.

Inhibition of binding of the peptides to lectins by mucin and fetuin supported the hypothesis that the peptides were interacting at glycan-binding sites. Mucin, which contains oligosaccharides with terminal GalNAc, Gal, GlcNAc, fucose and a minor amount of Neu5Ac, had only a slight inhibitory effect on binding of peptides to SNA1 or MAA. However, fetuin, which has multiple glycans that terminate in Neu5Ac-Gal, strongly inhibited binding to SNA1 and MAA. These results, coupled with the relatively high affinity of the longer peptides for SNA1 and MAA, with half-maximal binding in the low nanomolar range, suggested that the peptides effectively mimic Neu5Ac-Gal.

Glycans are modulators of the activity of phagocytic cells. A soluble glucomannan isolated from *Candida utilis* primed phagocytic cells to respond to opsonized pathogens.[39] Glucomannan is a polymer of about 350 monosaccharide residues and is active in the range of 1 mg/ml, equivalent to a monosaccharide concentration of about 5 mM. Relatively high concentrations of fetuin attenuated the inflammatory action of lipopolysaccharide on macrophages.[40] Fetuin also stimulated phagocytosis by macrophages at concentrations of 10 to 20 μM.[41,42] Other factors activate phagocytic cells at very low concentrations. For example, a macrophage activating factor was characterized that contains a single GalNAc residue that is required for its activity.[43] This glycoprotein is active *in vitro* at concentrations near 2 pM.[44] Other glycan-containing molecules influence endocytic activity at concentrations between these extremes.[1,45,46] However, we wish to state that although the peptides behave as glycomimetics, they may engage in simply peptide-protein interactions and not mimic glycan-receptor interactions.

We considered the possibility that the peptides would stimulate phagocytosis. When incubated with PBMCs, the longer peptide NPSHPLSG did not stimulate the release of inflammatory cytokines such as IL-1α. IL-1β, IL-2, IL-6, IL-8, TNF-α or IFN-γ[8] that often accompanies classical activation of macrophages, or IL-4 and IL-13, which are involved in the alternative activation pathway.[38] Yet opsonized microspheres were actively internalized by adherent cells when treated with nanomolar concentrations of the peptide. As shown in Figure 8, the three peptides strongly stimulated internalization of the beads. Current models suggest that interaction of cytoplasmic domains of Fcγ receptors result in tyrosine phosphorylation of ITAM (immunoreceptor tyrosine-based activating motif) domains.[36] The quadravalent structure of the

peptides was designed to facilitate the cross-linking of receptors required for this interaction. The signal-transduction pathway for activation of phagocytosis includes downstream phosphorylation of phosphoinositol-3 kinase (PI3K), which is specifically inhibited by wortmannin.[36] The essentially complete inhibition of uptake of microspheres by wortmannin supported the hypothesis that the peptides stimulate phagocytosis through the PI3K pathway. This pathway is also involved in the non-inflammatory, alternative activation of macrophages,[38] which is consistent with the stimulation of STAT6 phosphorylation by the peptides (Fig. 10). The discriminatory binding of the longer peptides to lectins, the higher affinity afforded by the multivalent structure, and the absence of release of inflammatory cytokines suggests that these synthetic peptides could be used to selectively activate cell receptors and may be useful as biological response modifiers.

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## Conflicts of Interest

LLE and JKH declare that they are inventors of the technology contained in this report. Intellectual property has been assigned to Susavion Biosciences, Inc., in which the authors hold shares.

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